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<b>(54) Title:</b> ISOLATION OF PRECURSOR CELLS FROM HEMATOPOIETIC AND NON-HEMATOPOIETIC TISSUES AND THEIR USE  <b>(57) Abstract</b>  Subsets of cells from hematopoietic and non-hematopoietic tissues are used for bone and cartilage regeneration. Bone and cartilage precursor cells are isolated from peripheral blood, bone marrow and adipose tissue using antibodies and other reagents that recognize hematopoietic cell surface marker CD34 and other markers on CD34+ cells. Precursors cells may be used for in vivo bone or cartilage regeneration by transplanting the cells with or without a carrier material to sides in the body requiring bone or cartilage repair without the need for in vitro culturing. Precursor cells may also be used to seed prosthetic devices to enhance implantability.		

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**ISOLATION OF PRECURSOR CELLS FROM HEMATOPOIETIC AND NON-HEMATOPOIETIC TISSUES AND THEIR USE****BACKGROUND OF THE INVENTION**

5           The present invention generally relates to the isolation of precursor cells and their use in bone and cartilage regeneration procedures and, more particularly, is concerned with a direct method for isolating precursor cells from a variety of body tissue  
10 types utilizing cell surface antigen CD34 and other precursor cell surface antigens on CD34+ cells.

Osteogenesis and chondrogenesis are highly complex biological processes having considerable medical and clinical relevance. For example, more than 1,400,000  
15 bone grafting procedures are performed in the developed world annually. Most of these procedures are administered following joint replacement surgeries, or during trauma surgical reconstructions. The success or failure of bone grafting procedures depends largely on  
20 the vitality of the site of grafting, graft processing, and in the case of allografts, on immunological compatibility between donor and host. Compatibility issues can largely be negated as an important consideration in the case of autologous grafting

procedures, which involve taking bone tissue from one site of the patient for transplantation at another site. While autologous bone grafts are generally successful they do require additional surgery in order  
5 to harvest the graft material, and not uncommonly are accompanied by post-operative pain, hemorrhage and infection.

Cartilage regeneration and replacement procedures are perhaps even more problematic. Unlike osteogenesis,  
10 chondrogenesis does not typically occur to repair damaged cartilage tissue. Attempts to repair damaged cartilage in any clinically meaningful fashion have met with only limited success. In many cases, the most effective treatment for cartilage damage is prosthetic  
15 joint replacement.

These and other difficulties with presently available bone-grafting and cartilage regeneration procedures have prompted intensive investigations into the cellular and molecular bases of osteogenesis and  
20 chondrogenesis. Some promising research to date has been in the identification and isolation of bone and cartilage precursor cells from marrow and other tissues.

Early investigations into the complexity of bone marrow demonstrated that lethally irradiated animals could be rescued by marrow transplants, suggesting that bone marrow contained a restorative factor having the capacity to regenerate the entire hematopoietic system. More recent experiments have shown that marrow also has the capacity to regenerate bone and other mesenchymal tissue types when implanted in vivo in diffusion chambers. (See e.g. A. Friedenstein et al. "Osteogenesis in transplants of bone marrow cells." J. Embryol. Exp. Morph. 16, 381-390, 1960; M. Owen. "The osteogenic potential of marrow." UCLA Symp. on Mol. and Cell. Biol. 46, 247-255, 1987) Results of this nature have led to the conclusion that bone marrow contains one or more populations of pluripotent cells, known as stem cells, having the capacity to differentiate into a wide variety of different cell types of the mesenchymal, hematopoietic, and stromal lineages.

The process of biological differentiation, which underlies the diversity of cell types exhibited by bone marrow, is the general process by which specialized, committed cell types arise from less specialized, primitive cell types. Differentiation may conveniently

be thought of as a series of steps along a pathway, in which each step is occupied by a particular cell type potentially having unique genetic and phenotypic characteristics. In the typical course of

5 differentiation a pluripotent stem cell proceeds through one or more intermediate stage cellular divisions, ending ultimately in the appearance of one or more specialized cell types, such as T lymphocytes and osteocytes. The uncommitted cell types which

10 precede the fully differentiated forms, and which may or may not be true stem cells, are defined as precursor cells.

Although the precise signals that trigger differentiation down a particular path are not fully

15 understood, it is clear that a variety of chemotactic, cellular, and other environmental signals come into play. Within the mesenchymal lineage, for example, mesenchymal stem cells (MSC) cultured in vitro can be induced to differentiate into bone or cartilage in vivo

20 and in vitro, depending upon the tissue environment or the culture medium into which the cells are placed.

(See e.g. S Wakitani et al. "Mesenchymal cell-based repair of large, full-thickness defects of articular

cartilage" J. Bone and Joint Surg, 76-A, 579-592  
(1994); J Goshima, VM Goldberg, and AI Caplan, "The  
osteogenic potential of culture-expanded rat marrow  
mesenchymal cells assayed in vivo in calcium phosphate  
5 ceramic blocks" Clin. Orthop. 262, 298-311 (1991); H  
Nakahara et al "In vitro differentiation of bone and  
hypertrophic cartilage from periosteal-derived cells"  
Exper. Cell Res. 195, 492-503 (1991)).

Studies of this type have conclusively shown that  
10 MSC are a population of cells having the capacity to  
differentiate into a variety of different cell types  
including cartilage, bone, tendon, ligament, and other  
connective tissue types. Remarkably, all distinct  
mesenchymal tissue types apparently derive from a  
15 common progenitor stem cell, viz. MSC. The MSC itself  
is intimately linked to a trilogy of distinctly  
differentiating cell types, which include  
hematopoietic, mesenchymal, and stromal cell lineages.  
Hematopoietic stem cells (HSC) have the capacity for  
20 self-regeneration and for generating all blood cell  
lineages while stromal stem cells (SSC) have the  
capacity for self-renewal and for producing the  
hematopoietic microenvironment.

It is a tantalizing though controversial prospect whether the complex subpopulations of cell types present in marrow (i.e. hematopoietic, mesenchymal, and stromal) are themselves progeny from a common ancestor.

5 The search for ancestral linkages has been challenging for experimentalists. Identifying relatedness among precursor and stem cell populations requires the identification of common cell surface markers, termed "differentiation antigens," many of which appear in a

10 transitory and developmentally-related fashion during the course of differentiation. One group, for example, has reported an ancestral connection among MSC, HSC, and SSC, though later issued a partial retraction (S. Huang & L. Terstappen. "Formation of haematopoietic

15 microenvironment and haematopoietic stem cells from single human bone marrow stem cells" Nature, 360, 745-749, 1992; L. Terstappen & S. Huang. "Analysis of bone marrow stem cell" Blood Cells, 20, 45-63, 1994; EK Waller et al. "The common stem cell hypothesis

20 reevaluated: human fetal bone marrow contains separate populations of hematopoietic and stromal progenitors" Blood, 85, 2422-2435, 1995). However, studies by another group have demonstrated that murine osteoblasts



possess differentiation antigens of the Ly-6 family. That finding is significant in the present context because the Ly-6 antigens are also expressed by cells of the murine hematopoietic lineage. (M.C. Horowitz  
5 etal. "Expression and regulation of Ly-6 differentiation antigens by murine osteoblasts" Endocrinology, 135, 1032-1043, 1994) Thus, there may indeed be a close lineal relationship between mesenchymal and hematopoietic cell types which has its  
10 origin in a common progenitor. A final answer on this question must await further study.

One of the most useful differentiation antigens for following the course of differentiation in human hematopoietic systems is the cell surface antigen known  
15 as CD34. CD34 is expressed by about 1% to 5% of normal human adult marrow cells in a developmentally, stage-specific manner [CI Civin etal. "Antigenic analysis of hematopoiesis.III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised  
20 against KG-1a cells. J Immunol, 133, 157-165, 1984]. CD34+ cells are a mixture of immature blastic cells and a small percentage of mature, lineage-committed cells of the myeloid, erythroid and lymphoid series. Perhaps

1% of CD34+ cells are true HSC with the remaining number being committed to a particular lineage. Results in humans have demonstrated that CD34+ cells isolated from peripheral blood or marrow can reconstitute the entire hematopoietic system for a lifetime. Therefore, CD34 is a marker for HSC and hematopoietic progenitor cells.

While CD34 is widely recognized as a marker for hematopoietic cell types, it has heretofore never been recognized as a reliable marker for precursor cells having osteogenic potential in vivo. On the contrary, the prior art has taught that bone precursor cells are not hematopoietic in origin and that bone precursor cells do not express the hematopoietic cell surface antigen CD34 (MW Long, JL Williams, and KG Mann "Expression of bone-related proteins in the human hematopoietic microenvironment" J. Clin. Invest. 86, 1387-1395, 1990; MW Long et al. "Regulation of human bone marrow-derived osteoprogenitor cells by osteogenic growth factors" J. Clin. Invest. 95, 881-887, 1995; SE Haynesworth et al. "Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies" Bone, 13, 69-80, 1992).

To date, the most common sources of precursor cells having osteogenic potential have been periosteum and marrow. Many researchers use cells isolated from periosteum for in vitro assays (See e.g. I Binderman et al. "Formation of bone tissue in culture from isolated bone cells" J. Cell Biol. 61, 427-439, 1974). The pioneer of the concept of culturing bone marrow to isolate precursor cells for studying bone and cartilage formation is A.J. Friedenstein. He developed a culture method for isolating and expanding cells (CFU-f) from bone marrow which can form bone (AJ Friedenstein et al. "The development of fibroblast colonies in monolayer cultures of guinea pig bone marrow and spleen cells" Cell Tiss. Kinet. 3, 393-402, 1970). Others have used Friedenstein's culture system extensively to study the origin of osteoblasts (See e.g. M. Owen, "The origin of bone cells in the postnatal organism" Arthr. Rheum. 23, 1073-1080, 1980). Friedenstein showed that CFU-f cells from marrow will form bone, cartilage, and fibrous tissue when implanted, though CFU-f cells cultured from other sources such as thymus, spleen, peripheral blood, and peritoneal fluid will not form bone or cartilage without an added inducing agent. Friedenstein recently

discussed the possible clinical utility of CFU-f and pointed out some obstacles that must be overcome, such as the need for culturing for several passages and developing a method for transplanting the cells (AJ  
5 Friedenstein "Marrow stromal fibroblasts" Calcif. Tiss. Int. 56(S): S17, 1995).

Similarly, the most common sources of cartilage precursor cells to date have been periosteum, perichondrium, and marrow. Cells isolated from marrow  
10 have also been used to produce cartilage in vivo (S Wakitani et al. "Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage" J. Bone and Joint Surg, 76-A, 579-592 (1994).

Periosteal and perichondral grafts have also been used  
15 as sources of cartilage precursor cells for cartilage repair (SW O'Driscoll et al. "Durability of regenerated articular cartilage produced by free autogenous periosteal grafts in major full-thickness defects in joint surfaces under the influence of continuous  
20 passive motion" J. Bone and Joint Surg. 70A, 1017-1035, 1986; R Coutts et al. "Rib perichondral autografts in full-thickness articular defects in rabbits" Clin. Orthop. Rel. Res. 275, 263-273, 1992).

In a series of patents, Caplan et al. disclose a method for isolating and amplifying mesenchymal stem cells (MSC) from marrow. (U.S. Patents 4,609,551; 5,197,985; and 5,226,914) The Caplan method involves  
5 two basic steps: 1) harvesting marrow and 2) amplifying the MSC contained in the harvested marrow by a 2 to 3 week period of in vitro culturing. This method takes advantage of the fact that a particular culture medium favors the attachment and propagation of MSC over other  
10 cell types. In a variation on this basic method, MSC are first selected from bone marrow using specific antibodies against MSC prior to in vitro culturing. (Caplan and Haynesworth; WO 92/22584) The in vitro amplified, marrow-isolated MSC may then be introduced  
15 into a recipient at a transplantation repair site. (A. Caplan. "precursor cells cells" J. Ortho. Res. 9, 641, 1991; S.E.Haynesworth, M.A.Baber, and A.I. Caplan. "Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal  
20 antibodies," Bone, 13, 69-80, 1992)

The current methods used to isolate precursor cells have a number of drawbacks to consider. First, the methods require that bone marrow or other tissues

be harvested. Harvesting bone marrow requires an additional surgical procedure with the attendant possibility of complications from anesthesia, hemorrhage, infection, and post-operative pain.

5 Harvesting periosteum or perichondrium is even more invasive. Second, the Caplan method requires a substantial period of time (2 to 3 weeks) for in vitro culturing of marrow-harvested MSC before the cells can be used in further applications. This additional cell  
10 culturing step renders the method time-consuming, costly, and subject to more chance for human error.

Consequently, a need exists for a quicker and simpler method for identifying and isolating precursor cells having osteogenic and chondrogenic potential  
15 which can be used for in vivo bone and cartilage regeneration procedures.

#### SUMMARY OF THE INVENTION

It is an object of this invention to provide a  
20 method for isolating precursor cells having the potential to generate bone or cartilage from a variety of hematopoietic and non-hematopoietic tissues.

It is also an object of this invention to provide a method for isolating precursor cells having the potential to generate bone or cartilage from peripheral blood, marrow, or adipose tissue based on binding by a reagent to cell surface antigen CD34 or other surface antigens on CD34+ cells.

It is another object of this invention to provide a method for isolating precursor cells having the potential to generate bone or cartilage from adipose tissue based on sedimentation differences in the cells comprising the tissue.

It is a further object of the present invention to provide a method for in vivo bone and cartilage regeneration involving transplantation with CD34+ precursor cells isolated from peripheral blood, marrow, or adipose tissue.

It is a still further object of the present invention to provide a direct, single-step method for in vivo bone or cartilage regeneration involving the isolation of CD34+ precursor cells from peripheral blood, marrow, or adipose tissue and immediate implantation at a connective tissue site needing repair

without the need for in vitro culturing of precursor cells.

It is yet another object of the present invention to provide a method to enhance the implantability of  
5 bone prosthetic devices.

It is still another object of the present invention to provide an improved bone implantation prosthetic device in which the device is seeded with precursor cells having osteogenic potential isolated  
10 from a patient's peripheral blood, bone marrow, or adipose tissue.

These and other objects are provided by the present invention.

The ability to isolate autologous precursor cells  
15 having osteogenic and chondrogenic potential has far reaching clinical implications for bone and cartilage repair therapies, either alone or in conjunction with prosthetic devices. The present invention provides a simple method for isolating precursor cells having the  
20 potential to generate bone or cartilage from a variety of tissue types including peripheral blood, marrow, and adipose tissue. The precursor cells are isolated using reagents that recognize CD34 or other markers on the



surface of CD34+ precursor cells, for example CD33, CD38, CD74, and THY1. Significantly, the present invention does not require in vitro culturing of isolated precursor cells before the cells can be used  
5 in further in vivo procedures. Indeed, precursor cells isolated by the present invention may be transplanted in vivo immediately for bone or cartilage regeneration. Thus, the 2 to 3 week time delay required by other methods for in vitro culturing of progenitor cells is  
10 eliminated making the method economical, practical and useful for the clinical environment.

Accordingly, the present invention relates to a method for isolating precursor cells having the potential to generate bone or cartilage directly from  
15 hematopoietic and non-hematopoietic tissues, including peripheral blood. The method includes steps of collecting tissue samples, contacting the sample with an antibody or other reagent that recognizes antigen CD34 or other antigens on CD34+ precursor cells, and  
20 separating the reagent-precursor cell complex from unbound material, by for example, affinity chromatography. Precursor cells isolated by the present

method may be used immediately for bone and cartilage regeneration in vivo.

In one aspect, the present invention is a method for isolating precursor cells having the potential to  
5 generate bone or cartilage from peripheral blood, marrow or adipose tissue.

In another aspect, the present invention is a method for isolating precursor cells having the potential to generate bone or cartilage based on  
10 selecting cells from hematopoietic and non-hematopoietic tissues that carry cell surface marker CD34.

In yet another aspect, the present invention is a method for bone or cartilage regeneration which  
15 utilizes CD34+ precursor cells isolated from peripheral blood, marrow, or adipose tissue.

#### DESCRIPTION OF THE PREFERRED EMBODIMENT(S)

Terms used throughout this disclosure are defined  
20 as follows:

##### Adipose Tissue

A complex tissue containing multiple cell types including adipocytes and microvascular cells. Adipose

tissue is one of the most convenient sources of precursor cells in the body. As used herein the term "adipose tissue" is intended to mean fat and other sources of microvascular tissue in the body such as placenta or muscle. The term specifically excludes connective tissues, hematologic tissues, periosteum, and perichondrium.

#### Chondrogenic

The capacity to promote cartilage growth. This term is applied to cells which stimulate cartilage growth, such as chondrocytes, and to cells which themselves differentiate into chondrocytes. The term also applies to certain growth factors, such as TGF- $\beta$ , which promote cartilage growth.

#### 15 Connective Tissue

Any of a number of structural tissues in the body including bone, cartilage, ligament, tendon, meniscus, and joint capsule.

#### Differentiation

20 A biological process in which primitive, unspecialized, cells undergo a series of cellular divisions, giving rise to progeny having more specialized functions. The pathway to terminal

differentiation ends with a highly specialized cell having unique genetic and phenotypic characteristics. The conventional wisdom of the past taught that differentiation proceeded in one direction only - from less specialized to more specialized. This dogma is now being challenged by new results which suggest that in fact the pathway may be bi-directional. Under certain conditions more specialized cells may in fact produce progeny which effectively reverse the flow toward greater specialization.

#### Hematopoietic Stem Cell

Primitive cell having the capacity to self-renew and to differentiate into all blood cell types.

#### Mesenchymal Stem Cell

Primitive cell type having the capacity for self-regeneration and for differentiating through a series of separate lineages to produce progeny cells having a wide variety of different phenotypes, including bone, cartilage, tendon, ligament, marrow stroma, adipocytes, dermis, muscle, and connective tissue.

#### Microvascular Cell

Cells comprising the structure of the microvasculature such as endothelial, smooth muscle, and pericytes.

Osteogenic

The capacity to promote or to generate the production of bone. The term may be applied to osteoblasts which have the capacity to promote bone growth, or to cells which themselves are able to differentiate into osteoblasts. The term would also apply to growth factors having the capacity to promote bone growth.

Precursor Cell

A cell with the potential to differentiate to perform a specific function.

Stem Cell

Pluripotent precursor cell having the ability to self-renew and to generate a variety of differentiated cell types.

The present invention is premised upon two surprising discoveries. First, that precursor cells having the potential to form connective tissue in vivo can be isolated from a variety of hematopoietic and non-hematopoietic tissue sources, including peripheral blood, and adipose tissue. And second, that cell surface marker CD34, a heretofore unrecognized identifier for connective tissue precursor cells, may

be used as a marker for precursor cells having the potential to form bone and cartilage in vivo.

The inventors have discovered two convenient, new sources for precursor cells (viz. peripheral blood and  
5 adipose tissue), and a source from marrow which does not require in vitro culture. Unlike prior methods, which have used bone marrow or periosteum as the source for osteogenic and chondrogenic precursor cells, the present invention enables isolation of these cells from  
10 more conveniently harvested tissues, such as peripheral blood and adipose tissue. The ability to isolate osteogenic and chondrogenic precursor cells from tissues other than marrow and periosteum lends considerable convenience and simplicity to an otherwise  
15 complicated method.

In one embodiment, the present invention is an affinity method enabling the isolation of precursor cells in humans having the potential to generate connective tissue based on expression of antigen CD34  
20 and other cell surface markers on CD34+ cells. Some examples of other markers on CD34+ cells would include CD33, CD38, CD74, and THY1, which list is not intended to be exclusive. In another embodiment, precursor cells

are isolated from adipose tissue based on differential sedimentation properties. Significantly, unlike previous methods, the present invention enables the immediate use of isolated precursor cells for bone and cartilage regeneration procedures without the need for in vitro culturing. As a consequence, the present method is quicker and easier to implement than previously described procedures.

10

### ***I. Isolating Precursor Cells***

In one embodiment, the present method for isolating precursor cells involves collecting a body tissue sample, contacting the sample with an antibody or other reagent that recognizes and binds to an antigen on the surface of the precursor cells, and then separating the precursor cell-reagent complex from unbound material by, for example, affinity chromatography. The method can be applied to peripheral blood, marrow, or other tissues, including adipose tissue. For ease and simplicity of isolation, however, blood is the preferred source material since surgical procedures are not required.

15  
20

(a) Peripheral blood as the source of precursor cells

By way of example, about 1 unit of blood is taken by any suitable means, for example by syringe withdrawal from the patient's arm. A particularly attractive method in the clinical environment is

5 apheresis, which has the added advantage of removing red cells. Removal of red cells is not essential, although it does enhance the performance of the method and is preferred. Red cells may be removed from the sample by any suitable means, for example, lysis,

10 centrifugation, or density gradient separation. It is preferred that the sample also be anticoagulated by, for example, treatment with citrate, heparin, or EDTA.

The yield of precursor cells is expected to be about 0.1% to 0.5% of the population of nucleated blood

15 cells. Yields may vary, depending upon the health and age of the donor, and on the freshness of the sample. The yield may be dramatically increased by administering drugs or growth factors to the patient before blood collection. Although the method will work

20 on samples which have been stored under refrigeration, fresh samples are preferred.

A critical step in the procedure of isolating precursor cells from peripheral blood involves



contacting the blood sample with a reagent that recognizes and binds to a cell surface marker on CD34+ cells. Any reagent which recognizes and binds to CD34+ cells is within the scope of the invention. Suitable  
5 reagents include lectins, for example soy bean agglutinin (SBA), antibodies and attachment molecules such as L-selectin.

In the preferred embodiment the sample is contacted with an antibody against CD34. Either  
10 monoclonal (mAb) or polyclonal antibodies may be used. Methods for preparing antibodies directed against CD34 and other cell surface antigens on CD34+ cells are well known to those skilled in the art. Suitable human antibody preparations directed against CD34 and other  
15 cell surface markers on CD34+ cells may be obtained commercially from Cell Pro, Inc., Bothell, WA, or Becton-Dickinson, Mountain View, CA.

Suitable cell surface antigens on precursor cells include CD34 and other antigens on CD34+ cells, for  
20 example THY1, CD33, CD38, and CD74. The preferred cell surface marker is CD34. It is expected that the procedure will be successful using other cell surface antigens on CD34+ cells as markers for precursor cells.

Following a brief incubation of the sample with the antibody to enable binding, the precursor cell-antibody complex is recovered by any suitable method such as, for example, affinity chromatography, magnetic  
5 beads, and panning. In the preferred embodiment, recovery is by affinity chromatography. (see, e.g. RJ Berenson et al. "Positive selection of viable cell populations using avidin-biotin immunoadsorption" J. Immunolog. Meth. 91, 11-19, 1986)

10 Briefly, the affinity recovery method utilizes a biotin-avidin coupling reaction in which the antibody is coupled to biotin by any suitable method. The antibody-biotin labeled precursor cell complex is separated from unbound materials by passing the  
15 reaction mixture through a column packed with an avidin labeled matrix. Unbound materials are removed from the column by washing. A useful commercially available cell separation kit includes biotin-labeled human anti-CD34 and a column packed with an avidin labeled matrix  
20 ("CEPRATE?LC" available from CellPro, Inc. Bothell, WA).

Indirect labelling methods are also within the scope of the invention. For example, the primary

antibody could be directed against a precursor cell surface marker and a secondary antibody, labelled with biotin, directed against the primary antibody. Alternatively, the secondary antibody may be coupled to  
5 a suitable solid support material.

Negative selection schemes are also intended to be within the scope of the invention. Using a negative selection, the antibody, or other reagent, would be directed against a cell surface marker which is absent  
10 on CD34+ cells.

(b) Bone marrow as the source of precursor cells

The method disclosed above for isolating precursor cells from blood may be applied in essentially the same fashion to bone marrow. Bone marrow is collected by any  
15 suitable fashion, for example iliac crest aspiration. In the preferred embodiment the marrow is treated with an anticoagulant such as EDTA, heparin, or citrate and nucleated cells are separated from non-nucleated cells by any suitable means, for example by hemolysis or by  
20 density gradient centrifugation.

Precursor cells that express the CD34 cell surface antigen are isolated from marrow using a reagent that recognizes and binds to CD34 or to some other antigen

on the surface of CD34+ cells. Suitable reagents include antibodies, lectins, and attachment molecules. Bound cells are separated from unbound cells by affinity chromatography, magnetic beads, or by panning.

5        In the preferred embodiment, an antibody directed against CD34 is used in the binding reaction and bound cells are separated from unbound cells by affinity chromatography, as disclosed more fully in the examples which follow.

10

(c) Adipose tissue as the source of precursor cells

As defined at the beginning of this section, "adipose tissue" is used throughout this disclosure in a generic sense to mean fat and other tissue types  
15 (excluding connective tissues, hematologic tissues, periosteum, and perichondrium) which contain microvascular cells. Microvascular tissue, from which capillaries are made, is an integral part of the blood transport system and, as such, is ubiquitous throughout  
20 the body. Microvascular tissue is composed of at least three cell types - endothelial, pericytes, and smooth muscle. Early investigations suggested that microvascular tissue might play an important role in

bone metabolism. A key observation was that microvascular cells and tissue arose de novo and proliferated at sites of bone repair and new bone growth. Such observations led to speculation that endothelial cells, pericytes, or both may be osteoprecursor cells, or alternatively, that microvascular cells exert a mitogenic effect on bone precursor cells. (See e.g. C Brighton et.al. "The pericyte as a possible osteoblast progenitor cell" Clin. Orthop. 275, 287-299, 1992) A more recent study using in vitro cultured cells suggests both progenitor-like cell proliferation and mitogenic effects by microvascular cells. (AR Jones et.al. "Microvessel endothelial cells and pericytes increase proliferation and repress osteoblast phenotype markers in rat calvarial bone cell cultures" J. Ortho. Res. 13, 553-561, 1995). Thus, within the microvascular cell population are precursor cells having osteogenic and chondrogenic potential.

The method of the present invention, as applied to adipose tissue, has two embodiments. In the first embodiment, the tissue is contacted with a reagent that recognizes CD34 or other surface antigen on CD34+

cells. As with peripheral blood and marrow, suitable binding reagents for use with adipose tissue include lectins, antibodies, and attachment molecules. The affinity binding method, as applied to adipose tissue, differs from the method as applied to blood and marrow by requiring a step to produce a single-cell suspension before incubation with the antigen binding reagent. Any suitable dissociation enzyme such as, for example, collagenase may be used. Cells that bind the reagent can be removed from unbound cells by any suitable means, for example affinity chromatography, magnetic beads, or panning.

In the preferred embodiment of the invention as applied to adipose tissue, a sedimentation method is utilized to obtain a fraction of cells that is enriched for precursor cells having osteogenic and chondrogenic potential. Following harvest of the tissue and digestion with an enzyme to form a single-cell suspension, the cells are separated by gravity sedimentation on the bench top, or by centrifugation.

By way of example, fat could be secured by liposuction or any other suitable method. About 10 cc to 30 cc of fat tissue is digested with enough

dissociation enzyme (e.g. collagenase) to produce a single-cell suspension. Suitable reaction conditions for enzyme digestion will vary depending on the enzyme used, as known to those skilled in the art. Following  
5 enzyme digestion, the adipocytes are separated from other cell types by centrifugation. Adipocytes float to the surface while denser cells, which include precursor cells, collect on the bottom and are separable thereafter by any suitable means. After washing the  
10 harvested precursor cells they can be mixed with a suitable carrier and immediately implanted in vivo at a site needing repair.

## **II. *In Vivo Mesenchymal Tissue Regeneration***

15 The precursor cells recovered by the present procedure are useful for a variety of clinical applications. For example, they may be transplanted without further processing to a connective tissue site in a patient to promote the repair or regeneration of  
20 damaged bone or cartilage.

Unlike previous methods, the present invention does not require in vitro culturing in order to obtain a suitable cell type or an adequate quantity of

precursor cells to be of use for in vivo application.

The present invention takes advantage of the unexpected finding that osteogenic and chondrogenic precursor cells may be isolated from a variety of hematopoietic  
5 and non-hematopoietic body tissues such as peripheral blood and adipose tissue. This finding has created a heretofore unappreciated reservoir of precursor cells that can be drawn from conveniently to provide enough cells for in vivo applications without an additional  
10 time-consuming step of amplifying cell numbers by in vitro culturing. This aspect of the invention saves time and money with less risk of complication and pain for the patient.

By way of example only and in no way as a  
15 limitation on the invention, the precursor cells isolated by the present method from any suitable tissue source may be implanted at any connective tissue site needing bone or cartilage regeneration. Suitable implanting procedures include surgery or arthroscopic  
20 injection.

While the factors that determine biological differentiation are not fully understood, it is known that precursor cells will differentiate into bone or



cartilage if transplanted to a site in the body needing repair. Precursor cells isolated by the present method can be implanted alone or premixed with growth factors such as TGF- $\beta$ . It is preferred that the cells be mixed  
5 with a suitable carrier material, well known to those skilled in the art, so as to impede the dislodgement of implanted cells. A non-exclusive list of suitable carriers would include, for example, proteins such as collagen or gelatin; carbohydrates such as starch,  
10 polysaccharides, saccharides, methylcellulose, agar, or algenate; proteoglycans; synthetic polymers; ceramics; or calcium phosphate.

The data presented in Table 2 demonstrate the operability of the invention for in vivo applications.  
15 The rat calvarial model used in these studies demonstrated that CD34+ cells isolated from marrow using a monoclonal antibody were as effective at promoting bone growth in an in vivo environment as were the positive controls (autologous graft). The data also  
20 show that the antibody itself can affect the outcome of the results probably via interaction with the complement system. For example, cells bound by mAb 5E6 did not stimulate bone growth in the rat calvarial

model. Although both antibodies tested recognize CD34 and are IgM isotypes, 5E6 binds complement effectively while 2C6 does not.

5

### III. Prosthetic Devices

A variety of clinically useful prosthetic devices have been developed for use in bone and cartilage grafting procedures. (see e.g. Bone Grafts and Bone Substitutions. Ed. M.B.Habal & A.H. Reddi, W.B. Saunders Co., 1992) For example, effective knee and hip replacement devices have been and continue to be widely used in the clinical environment. Many of these devices are fabricated using a variety of inorganic materials having low immunogenic activity, which safely function in the body. Examples of synthetic materials which have been tried and proven include titanium alloys, calcium phosphate, ceramic hydroxyapatite, and a variety of stainless steel and cobalt-chrome alloys. These materials provide structural support and can form a scaffolding into which host vascularization and cell migration can occur.

15  
20

Although surface-textured prosthetic devices are effectively anchored into a host as bare inorganic

structures, their attachment may be improved by seeding with osteogenic precursor cells, or growth factors which attract and activate bone forming cells. Such "biological-seeding" is thought to enhance the effectiveness and speed with which attachment occurs by providing a fertile environment into which host vascularization and cell migration can occur.

The present invention provides a source of precursor cells which may be used to "seed" such prosthetic devices. In the preferred embodiment precursor cells are first mixed with a carrier material before application to a device. Suitable carriers well known to those skilled in the art include, but are not limited to, gelatin, collagen, starch, polysaccharides, saccharides, proteoglycans, synthetic polymers, calcium phosphate, or ceramics. The carrier insures that the cells are retained on the porous surface of the implant device for a useful time period.

A more complete understanding of the present invention can be obtained by referring to the following illustrative examples of the practice of the invention, which examples are not intended, however, to be unduly limitative of the invention.

**EXAMPLE 1**Animal model for bone regenerating capacity of precursor cells

5           A rat calvarial model was used to test the operability of the invention for in vivo applications. The model consisted of monitoring the ability of various test samples to promote bone growth in calvarial defects which had been surgically introduced  
10 into the rat skull. Calvarial defects were introduced into 6 month to 9 month old Fisher rats having bodyweights in the range of about 300 g to 500 g according to the following procedure. Animals were anesthetized by intramuscular injection using a  
15 Ketamine- Rompun (xylazine)- Acepromazine (acepromazine maleate) cocktail, and surgical incisions made in the calvarial portion of the skull. After peeling back the skin flap, a circular portion of the skull measuring 8 mm in diameter was removed using a drill with a  
20 circular trephine and saline irrigation. An 8 mm diameter disk of "GELFILM" was placed in each defect to separate the exposed brain from the test material and to maintain hemostasis. The calvarial defects produced

in this fashion were then packed with a test sample consisting of an isolated cell population. For some experiments the test samples were mixed with a carrier material consisting of rat tail collagen or "AVITENE" 5 bovine collagen before introduction into the calvarial defect. The positive control consisted of an autograft while the negative control consisted of a tricalcium phosphate (TCP) carrier only implant. After surgical closure of the wound site, treated animals were 10 returned to their cages, maintained on a normal food and water regime, and sacrificed 28 days after surgery.

The effectiveness of a test sample to induce bone growth in calvarial defects was assessed by estimating new bone formation at the site of the defect by 15 measuring the closure in the linear distance between cut bone edges or noting islands of bone growth in the central portion of the defect. The scoring criteria are shown in Table 1. The results are summarized in Table 2.

20

## EXAMPLE 2

Isolation of an enriched nucleated cell population from rat bone marrow.

Rat bone marrow was isolated from the intramedullary cavities of 6 femurs taken from male Fisher rats between 8 to 10 weeks of age. Prior to sacrifice the animals had been maintained on a normal food and water diet. The marrow was extracted from excised femurs by flushing into a test tube containing approximately 5 ml of ACD buffer. Buffer ACD in the neat state consists of 2.2g  $\text{Na}_3\text{Citrate} \cdot 2\text{H}_2\text{O}$ , 0.8g citric acid, and 2.4g dextrose dissolved in 100 ml distilled water. Unless otherwise noted, buffer ACD was diluted to a concentration of 15% in PBS. The extracted marrow cells were gently suspended into the buffer solution by pipetting. In order to separate red cells from white cells, the marrow cell suspension was underlaid with approximately 4 ml of Ficoll-Hypaque with a specific gravity of 1.09 (Sigma Chemical Co., St. Louis, MO) and centrifuged at 1200 x g for 20 minutes. After centrifugation the interface layer containing the nucleated cells was removed by pipetting. The cells were washed in 5 ml of ACD and centrifuged at 250 x g for 6 to 7 minutes. The pellet was washed twice more in 1% BSA/PBS (bovine serum albumin, phosphate buffered saline; supplied with

CEPRATE LC kit). All PBS was Ca<sup>2+</sup> and Mg<sup>2+</sup> free to prevent clotting.

### EXAMPLE 3

Isolation of CD34+ cells from rat bone marrow using a  
5 monoclonal antibody and affinity chromatography and  
their use for in vivo bone regeneration in rat  
calvarial model.

#### Materials and Methods.

Mouse IgM monoclonal antibodies 2C6 and 5E6 were  
10 raised against rat CD34 present on the surface of a  
subpopulation of rat hematopoietic cells. The CD34  
mAb's used in these experiments were the gift of Dr.  
Othmar Forster and were prepared in a manner well-known  
to those skilled in the art. Anti-mouse IgM:FITC, used  
15 for fluorescence sorting of cells bound with mAb's 2C6  
and 5E6, was obtained from Boehringer Mannheim, Cat. #  
100807. Avidin:FITC also used in fluorescence sorting  
was obtained from Boehringer Mannheim, Cat. # 100205.  
CD34+ cells labeled with mAb 2C6 or 5E6 were separated  
20 from unbound cells using an affinity column method. A  
useful, commercially available affinity cell separation  
kit, "CEPRATE LC," may be obtained from CellPro  
(CellPro, Inc. Bothell, WA 98021). Anti-mouse

IgM:biotin was purchased from Southern Biotech, Birmingham, AL, Cat. # 1022-08.

Cells carrying the CD34 surface antigen were isolated from rat marrow as follows. The rinsed  
5 nucleated cells, isolated in the manner described in Example 2, were resuspended in about 0.5 ml of 1%BSA/PBS (from CellPro kit). Then, a volume of mAb ranging in concentration from about 1 µg/ml to 40 µg/ml was added and the mixture incubated for about 1 hour at  
10 room temperature with occasional, gentle agitation. Following incubation the mixture was brought to 10 ml with 1%BSA/PBS and the mixture centrifuged at 250 x g for 6 minutes. The pellet was gently resuspended and rinsed two additional times in 10 ml 1%BSA/PBS and spun  
15 as before. After another resuspension and centrifugation, the final cell pellet was resuspended in 2 ml 1%BSA/PBS for incubation with a biotinylated anti-mouse IgM.

About 10 µl of Goat anti-mouse IgM:biotin (0.5  
20 mg/ml before dilution) was added to the resuspended mAb-cell pellet obtained at the previous step. The mixture was incubated at room temperature for about 30 minutes with gentle agitation, after which the cells were



rinsed twice by centrifugation and resuspension in BSA/PBS, as previously described. The final cell pellet was resuspended to about  $100 \times 10^6$  cells/ml in 5% BSA in a volume of 1 ml to 4 ml for loading onto an avidin  
5 column.

Antibody-labeled and unlabeled cells were separated on the "CEPRATE LC" avidin column using the conditions recommended by the manufacturer (Cell Pro, Inc., Bothell, WA). Briefly, the column contained a bed  
10 of PBS- equilibrated avidin matrix. Prior to loading the sample, about 5 ml of 5% BSA was run through the column. The pre-diluted cell sample was then layered onto the top of the gel matrix and the sample thereafter allowed to run into the matrix gel.

15 Unlabeled cells were washed from the column with about 3 ml to 5 ml of PBS. The mAb-labeled cells were then released from the matrix and collected into a small volume of 5% BSA by gently squeezing the column so as to agitate the matrix while washing the column with  
20 PBS. Small aliquots were saved from the bound and unbound fractions for cell counting and flow cytometry. For implantation experiments the cells were washed 2 times in PBS/BSA and once in PBS only.

*Results.*

Each experiment generated about 10 to 20 x 10<sup>6</sup> adherent cells of which about half this number were implanted into a calvarial defect. Cell fractions taken  
5 from the column were tested for viability by trypan blue cell counts using a hemacytometer and found to be in the range of about 85% to 97% viable. The adherent cell population appeared to be a group of small blast cells. FACS was used to determine the purity of CD34+  
10 cells isolated on the column. The adherent cell population contained about 50% of the original number of CD34+ cells at a purity of about 50%.

CD34+ cells were implanted into rat calvarial defects with or without a suitable carrier material.  
15 Two carriers were tried in these experiments, "AVITENE" and rat tail collagen, both of which were found to be useful. Rat tail collagen is preferred, however, since it showed the least inflammatory response. About 50 mg of collagen was dissolved in 1 ml of PBS at 60°C and  
20 equilibrated to 37°C prior to mixing with cells. In some experiments pellets containing collagen and cells were formed by mixing 100 µl of collagen solution with a cell pellet and cooling the mixture to 4°C prior to

implantation into a calvarial defect. Surgical implantations were performed as described in Example 1 with sacrifice of recipient animals at 28 days post-surgery.

5 Histology scoring for bone formation was assessed according to the scheme shown in Table 1.

*Discussion.*

The finding that CD34+ cells isolated by mAb 5E6 failed to stimulate bone regeneration in vivo may be  
10 explained by the ancillary observation that this antibody is a more effective activator of the complement system than mAb 2C6 (data not shown).

**EXAMPLE 4**

15 (a) Bone regeneration in rat calvarial model using Ficoll-separated whole blood.

The rat calvarial model described in Example 1 was used to determine the bone regenerating capacity of Ficoll-separated whole blood. Approximately 2.5 ml of  
20 donor blood was used for each recipient calvarial defect. Donor animals were 8 to 10 week old male F344 strain rats. Recipients were 6 to 8 months old. Donors

were bled into 3 cc syringes, which contained about 0.5 cc of ACD solution to inhibit coagulation.

ACD Stock Solution \_\_\_\_\_ ACD Working Solution

2.2 g Na<sub>3</sub>Citrate.2H<sub>2</sub>O 15 ml ACD Stock Solution

5      0.8 g citric acid.1H<sub>2</sub>O      100 ml PBS (Ca<sup>++</sup>/Mg<sup>++</sup> free)

2.4 g dextrose

100 ml distilled water

Blood was placed into 15 ml conical tubes and brought up to 5 ml with ACD working solution. The  
10 samples were underlaid with 4 ml of Ficoll-Hypaque and centrifuged at 1200 xg at room temperature for 20 minutes. After centrifugation, the white cell layer was removed from each tube by pipet.

Ficoll-separated blood cells were used for  
15 implantation experiments, either directly or after mixing with a carrier material. For direct implantation, the cell pellet was washed twice in 10 ml of PBS and the final pellet, containing roughly 5 to 10 x 10<sup>6</sup> cells, delivered neat into a calvarial defect.  
20 Cell samples pre-mixed with a carrier material were combined with rat tail collagen prior to implantation. About 50 mg of rat tail collagen (obtained from Sigma, St. Louis, MO; Cat.# C-8897) was heated to 60°C in 500

μl PBS to dissolve the collagen protein. The collagen solution was equilibrated to 37°C prior to mixing with the cell pellet. About 60μl of collagen solution was mixed with the cell pellet and the entire cell-collagen mixture implanted into a calvarial defect.

#### EXAMPLE 5

Isolation of CD34+ cells from rat blood using a monoclonal antibody and affinity chromatography.

10 (1) Hemolysis Buffer - 10X Stock Solution

Dissolve the following in 1 L distilled water, adjust pH to 7.3, filter sterilize and store at 2 - 8°C.

83 g NH<sub>4</sub>Cl

10 g NaHCO<sub>3</sub>

15 4 g Na<sub>2</sub>EDTA

(2) Phosphate Buffered Saline (PBS) Ca<sup>2+</sup> and Mg<sup>2+</sup>

Free

Dissolve in 1 L distilled water, adjust pH to 7.2,  
20 filter sterilize, and store at 2 - 8°C.

8 g NaCl

1.15 g Na<sub>2</sub>HPO<sub>4</sub>

0.2 g KH<sub>3</sub>PO<sub>4</sub>

0.2 g KCl

(3) PBS + Bovine Serum Albumin

Dissolve 1g BSA in 100 ml PBS.

5       (a) Approximately 100 ml of whole blood was  
collected by cardiac puncture from 17 male F344 rats 8  
to 10 weeks old and heparinized by standard procedures.  
Red cells were lysed by mixing the whole blood with 300  
ml of 1X hemolysis buffer at 37°C and allowing the  
10 mixture to sit for about 3 minutes. Then 100 ml of  
PBS/BSA washing solution was added and the mixture  
centrifuged at 170 xg for 10 minutes. The resulting  
supernatant was aspirated without disturbing the cell  
pellet. The pellet was washed two more times by gently  
15 resuspending in PBS/BSA followed by centrifugation. The  
final pellet was brought up to 2 ml in PBS/BSA in  
preparation for incubation with the mAb, and a small  
aliquot removed for cell counting and FACS analysis.

(b) The cell pellet, resuspended in 2 ml PBS/BSA  
20 as in step (a), was incubated with 3 ml of neat mAb 2C6  
in order to bind CD34+ cells. The mAb-cell mixture was  
incubated at 4°C for 45 minutes and the cells gently  
agitated once to resuspend during incubation. Following

the incubation period the volume was brought up to 10 ml with PBS/BSA and the sample washed twice as in step (a). The washed pellet was resuspended in 2 ml PBS/BSA and 15 ml of goat anti-mouse IgM:biotin was added for a 5 30 minute incubation at 4°C with one gentle agitation during incubation to resuspend cells. The cells were rinsed twice in PBS/BSA, as described in step (a), and the final pellet resuspended in 10 ml of 5% BSA. 5 ml of the resuspended pellet were used for each of two 10 "CEPRATE LC" column sorts, as described in Example 3. Antibody-bound cells were released from the column as described in Example 3 and the released cells washed twice in PBS/BSA, and once in PBS. The final cell pellet was mixed on a glass slide with 60ml of rat tail 15 collagen (100 mg/ml) at 37°C, and the mixture of collagen and cells placed briefly on ice to form a solid pellet. The cell-containing pellet was then transplanted immediately into a rat calvarial defect, as described in Example 1.

20

**EXAMPLE 6**

Isolation of microvascular cells from rat epididymal fat pads.

Two epididymal fat pads were removed by dissection from a male Fisher F344 rat, minced with scissors under sterile conditions, and incubated in 10 ml PBS/1%BSA in the presence of 8 mg/ml collagenase (Type II Crude, 5 273U/mg; Worthington Laboratories) for 45 minutes at 37°C with gentle shaking. After digestion the sample was centrifuged at 250 xg for 4 minutes and the low density fat at the top of the tube removed by aspiration. The pellet, which contained the precursor 10 cells, was washed twice in PBS/1%BSA and once in PBS. The washed pellet was mixed with 50 ml rat tail collagen at 37°C, placed briefly on ice to gel, and implanted into a rat calvarial defect.

It is thought that the method for isolating and 15 using bone and cartilage precursor cells by the present invention and many of its attendant advantages will be understood from the foregoing description and it will be apparent that various changes may be made in the form, construction, and arrangement of the elements 20 thereof without departing from the spirit and scope of the invention or sacrificing all of its material advantages, the form hereinbefore described being merely a preferred or exemplary embodiment thereof.



Table 1

## Bone Formation Scoring

	Site	Score	Description
5	Defect	0	No net gain in bone; either less formation than resorption or no formation at all.
10		1	Less than 5% of linear distance between cut bone edges is bridged by new bone.
15		2	About 5% to 33% of the defect is bridged by new bone, or there is an island of bone in the central portion of the defect.
		3	About 33% to 66% of the defect is bridged by new bone.
		4	Greater than 66% of the defect is bridged by new bone.
20		5	Complete bridging of the defect by new bone.

Table 2.

		RBRA	
Tissue/Cell Type		N	(Mean $\pm$ S.D.)
5			
	Autologous Graft (positive control)	142	2.4 $\pm$ 0.7
	TCP (negative control)	105	1.0 $\pm$ 0.9
	Marrow	30	2.5 $\pm$ 1.1
	Marrow Ficoll	18	2.3 $\pm$ 0.8
10	Marrow/Avitene	9	1.8 $\pm$ 0.4
	Blood Ficoll	11	1.3 $\pm$ 0.5
	Blood/RTC Ficoll	16	1.4 $\pm$ 0.5
	2C6+ cells	12	1.8 $\pm$ 0.4
	2C6- cells	12	0.7 $\pm$ 0.5
15	5E6+ cells	12	1.3 $\pm$ 0.6
	5E6- cells	12	1.5 $\pm$ 0.5
	SBA+ cells	12	1.8 $\pm$ 1.1
	SBA- cells	18	1.4 $\pm$ 0.7

20 RBRA: Relative bone regeneration activity

N: Number of experiments

S.D.: Standard deviation

2C6 and 5E6 cells were isolated from marrow

SBA: Soy Bean Agglutinin

We claim:

1. A method for isolating precursor cells for use in connective tissue formation, comprising the steps of:

a) collecting by any suitable means a body tissue  
5 containing said cells, wherein said body tissue is peripheral blood or bone marrow;

b) contacting said body tissue with a reagent that recognizes and binds to a cell surface marker on said cells, forming a cell-reagent complex wherein said  
10 reagent is a lectin or attachment molecule, and wherein said cell surface marker is an antigen selected from the group consisting of CD34 and antigens on the surface of CD34+ cells; and

c) separating said cell-reagent complex from said  
15 tissue by any suitable means.

2. A method, as in claim 1 wherein said separating step comprises a procedure selected from the group consisting of affinity chromatography, magnetic beads,  
20 and panning.

3. A method, as in claim 1 wherein said reagent is a lectin.

4. A method, as in claim 1 wherein said reagent is attachment molecule L-selectin.

5. A method, as in any one of claims 1 to 4 wherein  
5 said tissue is peripheral blood.

6. A method, as in any one of claims 1 to 4 wherein said tissue is bone marrow.

10 7. A method for isolating from peripheral blood precursor cells having osteogenic and chondrogenic potential for use in bone or cartilage formation, comprising the steps of:

- a) collecting said blood by any suitable means;
- 15 b) contacting said blood with a reagent comprising a lectin, antibody or attachment molecule wherein said reagent recognizes and binds to cell surface markers other than CD34 on CD34+ cells, forming a cell-reagent complex; and
- 20 c) separating said cell-reagent complex from said tissue by any suitable means.

8. A method, as in claim 7 wherein said reagent is an antibody and wherein said collecting step comprises apheresis.

5 9. A method for isolating a subpopulation of CD34+ precursor cells from peripheral blood having osteogenic and chondrogenic potential for use in bone or cartilage formation, comprising the steps of:

- a) collecting said blood by any suitable means;
- 10 b) contacting said blood with a reagent said reagent comprising a lectin, antibody or attachment molecule wherein said reagent recognizes and binds to cell surface markers other than CD34 on CD34+ cells, forming a cell-reagent complex; and
- 15 c) separating said cell-reagent complex from said tissue by any suitable means.

10. A method, as in claim 9 wherein said reagent is an antibody.

20

11. A method, as in claim 7, wherein said collecting step further comprises:

a) anticoagulating said blood by treatment with a reagent selected from the group consisting of citrate, EDTA, and heparin; and

b) removing red cells from said blood by any  
5 suitable means.

12. A method for isolating precursor cells from bone marrow for use in bone or cartilage formation, comprising the steps of:

10 a) collecting said marrow by any suitable means;  
b) contacting said marrow with a reagent that recognizes and binds to a cell surface marker on CD34+ cells, forming a cell-reagent complex; and  
c) separating said CD34+ cell-reagent complex from  
15 said marrow by any suitable means.

13. A method, as in claim 12 wherein said collecting step further comprises:

a) separating nucleated from non-nucleated cells  
20 by any suitable means; and  
b) treating with an anti-coagulant wherein said anticoagulant is EDTA, heparin, or citrate.

14. A method, as in claim 13 wherein said step of separating nucleated from non-nucleated cells comprises density gradient centrifugation or red blood cell lysis.

5

15. A method, as in claim 12 wherein said reagent is a lectin, antibody, or attachment molecule.

16. A method, as in claim 12 wherein said reagent is an  
10 antibody, said cell surface marker is CD34, and said separating means comprises affinity chromatography.

17. A method for isolating precursor cells from adipose tissue for use in bone or cartilage formation,  
15 comprising the steps of:

a) collecting said adipose tissue by any suitable means;

b) producing a single-cell suspension of said tissue by enzymatic dissociation;

20 c) contacting said single-cell suspension with a reagent that recognizes and binds to a cell surface marker on said cells forming a cell-reagent complex; and

d) separating said cell-reagent complex from said tissue by any suitable means.

18. A method, as in claim 17 wherein said reagent is an  
5 antibody, a lectin, or an attachment molecule and  
wherein said separating step comprises affinity  
chromatography, magnetic beads, or panning.

19. A method, as in claim 18 wherein said reagent is an  
10 antibody and wherein said cell surface marker is an  
antigen selected from the group consisting of CD34 and  
antigens on the surface of CD34+ cells.

20. A method for isolating precursor cells from adipose  
15 tissue for use in bone or cartilage formation,  
comprising the steps of:

- a) collecting said tissue by any suitable means;
- b) producing a single-cell suspension of said  
tissue by enzymatic dissociation; and
- 20 c) sedimenting said precursor cells from said  
single-cell suspension by any suitable means.



21. A method, as in claim 20 wherein said sedimenting step comprises centrifugation or gravitational sedimentation.

5 22. A method, as in claim 21 wherein said tissue comprises fat and wherein said collecting step comprises liposuction or surgery.

23. A negative selection method for isolating precursor  
10 cells for use in bone or cartilage formation from a hematopoietic or non-hematopoietic body tissue, comprising the steps of:

- a) collecting said tissue by any suitable means;
- b) contacting said tissue with a reagent that  
15 recognizes and binds to a cell surface marker, forming a cell-reagent complex, wherein said cell surface marker is excluded from the population of CD34+ cells;
- c) separating said cell-reagent complex from said tissue by any suitable means so that the fraction of  
20 cells remaining unbound contains an enriched population of said precursor cells.

24. A method, as in claim 23 wherein said tissue is peripheral blood or bone marrow.

25. A method, as in claim 24 wherein said reagent is an  
5 antibody, lectin, or attachment molecule.

26. A population of precursor cells having osteogenic and chondrogenic potential, said cells having been isolated from peripheral blood or adipose tissue.

10

27. A population of precursor cells having osteogenic and chondrogenic potential, said cells having been isolated from adipose tissue.

15 28. A population of precursor cells having osteogenic and chondrogenic potential, said cells having been isolated from bone marrow without an in vitro culturing step.

20 29. A population of precursor cells having osteogenic and chondrogenic potential, said cells having been isolated from peripheral blood as in any one of claims 1, 7, 8, or 11.

30. A population of precursor cells having osteogenic and chondrogenic potential, said cells having been isolated from adipose tissue as in any one of claims  
5 17, 18, 19, 20, or 22.

31. A population of precursor cells having osteogenic and chondrogenic potential, said cells having been isolated from bone marrow without an in vitro culturing  
10 step as in any one of claims 12, 14, 15, or 16.

32. A composition of matter, comprising:  
a) a population of precursor cells having osteogenic and chondrogenic potential as in any one of  
15 claims 26, 27, or 28; and  
b) a carrier material.

33. A composition of matter as in claim 32 wherein said carrier material is a protein, carbohydrate, synthetic  
20 polymer, or inorganic material.

34. A composition of matter, as in claim 33 wherein said carrier is a gelatin, collagen, polysaccharide,

saccharide, starch, proteoglycan, synthetic polymer, ceramic, or calcium phosphate.

35. A composition of matter, comprising:

- 5       a) a population of precursor cells having  
osteogenic and chondrogenic potential as in claim 29;  
and  
b) a carrier material.

10   36. A composition of matter, as in claim 35 wherein  
said carrier is a protein, carbohydrate, synthetic  
polymer, or inorganic material.

37. A composition of matter, as in claim 36 wherein  
15   said carrier is a gelatin, collagen, polysaccharide,  
saccharide, starch, proteoglycan, synthetic polymer,  
ceramic, or calcium phosphate.

38. A composition of matter, comprising:

- 20       a) a population of precursor cells having  
osteogenic and chondrogenic potential as in claim 30;  
and  
b) a carrier material.

39. A composition of matter, as in claim 38 wherein said carrier is a protein, carbohydrate, synthetic polymer, or inorganic material.

5

40. A population of precursor cells as in claim 39 wherein said carrier is a gelatin, collagen, polysaccharide, saccharide, starch, proteoglycan, synthetic polymer, ceramic, or calcium phosphate.

10

41. A composition of matter, comprising:

a) a population of precursor cells having osteogenic and chondrogenic potential as in claim 31; and

15       b) a carrier material.

42. A composition of matter, as in claim 41 wherein said carrier is a protein, carbohydrate, synthetic polymer, or inorganic material.

20

43. A composition of matter, as in claim 42 wherein said carrier is a gelatin, collagen, polysaccharide,

saccharide, starch, proteoglycan, synthetic polymer, ceramic, or calcium phosphate.

44. An implantable device useful for clinical  
5 applications, comprising:

a) at least one surface, having any suitable shape, said surface for contacting with a site in a patient requiring connective tissue repair;

b) said surface having been seeded with precursor  
10 cells having the potential to form connective tissue;  
and

c) wherein said cells have been isolated from peripheral blood or adipose tissue.

15 45. An implantable device as in claim 44 wherein said cells have been isolated from peripheral blood as in claim 1, 7, 10, or 11.

46. An implantable device as in claim 44 wherein said  
20 cells have been isolated from adipose tissue as in claim 17, 18, 19, 20, or 22.

47. An implantable device useful for clinical applications, comprising:

- a) at least one surface, having any suitable shape, said surface for contacting with a site in a patient requiring connective tissue repair;
- b) said surface having been seeded with precursor cells having the potential to form connective tissue; and
- c) wherein said cells have been isolated from bone marrow without an in vitro culturing step.

48. An implantable device as in claim 47 wherein said cells have been isolated from bone marrow as in claim 12, 14, 15, or 16.

15

49. An implantable device as in claim 44 or 47 wherein said surface comprises a textured region for connective tissue ingrowth.

20 50. An implantable device as in claim 44 or 47 wherein said cells are mixed with a carrier material.

51. An implantable device, as in claim 50 wherein said carrier is a protein, carbohydrate, synthetic polymer, or inorganic material.

5 52. An implantable device as in claim 50 wherein said carrier is a gelatin, collagen, polysaccharide, saccharide, starch, proteoglycan, synthetic polymer, ceramic, or calcium phosphate, and wherein said surface comprises a textured region for connective tissue  
10 ingrowth, said region comprising tricalcium phosphate, hydroxyapatite, alumina, titanium, titanium alloys, cobalt-chromium alloys, or stainless steel.

53. A method for enhancing the implantability of a  
15 prosthetic device at a site in a patient requiring bone regeneration, comprising the step of:

a) applying to a surface of said device precursor cells having the potential to form connective tissue, wherein said surface is for contacting with a site in a  
20 patient requiring bone repair; and

b) wherein said precursor cells have been isolated by any one of claims 7, 12, 19, 20, or 24.



54. A method for promoting mammalian bone regeneration in vivo using precursor cells taken directly from a body tissue containing said cells, comprising the steps of:

- 5       a) collecting said tissue by any suitable means;
- b) contacting said tissue with a reagent that recognizes and binds to a cell surface marker on said cells, forming a cell-reagent complex, wherein said cell surface marker is an antigen selected from the  
10   group consisting of CD34 and antigens on the surface of CD34+ cells;
- c) separating said cell-reagent complex from said tissue by any suitable means; and
- d) contacting, by any suitable means, said cell-  
15   reagent complex with a connective tissue site in a recipient requiring bone regeneration.

55. A method for promoting mammalian bone regeneration in vivo, as in claim 54 wherein said tissue is peripheral blood or bone marrow, and wherein said  
20   collecting step further comprises separating nucleated from non-nucleated cells by any suitable means and treating with an anticoagulant.

56. A method for promoting mammalian bone regeneration in vivo, as in claim 54 wherein said tissue is adipose tissue and wherein said collecting step further comprises forming a single-cell suspension by enzymatic dissociation.

57. A method for promoting mammalian bone regeneration in vivo, as in claim 55 or 56 wherein said reagent in step (b) comprises an antibody, a lectin, or an attachment molecule.

58. A method for promoting mammalian bone regeneration in vivo, as in claim 57 wherein said contacting step (d) comprises surgery or arthroscopic injection.

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59. A method for promoting mammalian bone regeneration in vivo, as in claim 58 wherein said contacting step (d) further comprises forming a mixture of said precursor cells with a carrier material selected from the group consisting of proteins, carbohydrates, synthetic polymers, or inorganic materials.

60. A method, as in claim 59 wherein said carrier material is a gelatin, collagen, polysaccharide, saccharide, starch, proteoglycan, synthetic polymer, ceramic, or calcium phosphate.

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61. A method for promoting mammalian bone regeneration in vivo using precursor cells taken directly from adipose tissue, comprising the steps of:

- a) isolating said cells as in claim 19 or 22; and
- 10       b) contacting, by any suitable means, said cells with a connective tissue site in a recipient requiring bone regeneration.

62. A method for promoting mammalian cartilage  
15 regeneration in vivo using precursor cells taken directly from a body tissue containing said cells, comprising the steps of:

- a) collecting said tissue by any suitable means;
- b) contacting said tissue with a reagent that  
20 recognizes and binds to a cell surface marker on said cells, forming a cell-reagent complex, wherein said cell surface marker is an antigen selected from the

group consisting of CD34 and antigens on the surface of CD34+ cells;

c) separating said cell-reagent complex from said tissue by any suitable means; and

5 d) contacting, by any suitable means, said cell-reagent complex with a connective tissue site in a recipient requiring cartilage regeneration.

63. A method for promoting mammalian cartilage regeneration in vivo, as in claim 62 wherein said  
10 tissue is peripheral blood or bone marrow, and wherein said collecting step further comprises separating nucleated from non-nucleated cells by any suitable means and treating with an anticoagulant.

15 64. A method for promoting mammalian cartilage regeneration in vivo, as in claim 62 wherein said tissue is adipose tissue and wherein said collecting step further comprises forming a single-cell suspension by enzymatic dissociation.

20

65. A method for promoting mammalian cartilage regeneration in vivo, as in claim 63 or 64 wherein said

reagent in step (b) comprises an antibody, a lectin, or an attachment molecule.

66. A method for promoting mammalian cartilage  
5 regeneration in vivo, as in claim 65 wherein said  
contacting step (d) comprises surgery or arthroscopic  
injection.

67. A method for promoting mammalian cartilage  
10 regeneration in vivo, as in claim 66 wherein said  
contacting step (d) further comprises forming a mixture  
of said precursor cells with a carrier material  
selected from the group consisting of proteins,  
carbohydrates, synthetic polymers, or inorganic  
15 materials.

68. A method, as in claim 67 wherein said carrier is a  
gelatin, collagen, polysaccharide, saccharide, starch,  
proteoglycan, synthetic polymer, ceramic, or calcium  
20 phosphate.

69. A method for promoting mammalian cartilage regeneration in vivo using precursor cells taken directly from adipose tissue, comprising the steps of:

- a) isolating said cells as in claim 19 or 22; and
- 5        b) contacting, by any suitable means, said cells with a connective tissue site in a recipient requiring cartilage regeneration.

70. A subpopulation of precursor cells having  
10        osteogenic and chondrogenic potential wherein said cells have been isolated from peripheral blood, adipose tissue, or marrow, without an in vitro culturing step.

71. A subpopulation of precursor cells having  
15        osteogenic and chondrogenic potential wherein said cells have been isolated from liposuction fat.

72. A method for promoting mammalian connective tissue regeneration using a subset of nucleated cells found in  
20        peripheral blood, comprising the steps of:

- a) isolating said cells by any suitable means; and

b) contacting by any suitable means said cells with a site in a patient requiring connective tissue repair.

5 73. A method for promoting mammalian connective tissue regeneration using a subset of cells found in adipose tissue, comprising the steps of:

a) isolating said cells by any suitable means; and

b) contacting by any suitable means said cells  
10 with a site in a patient requiring connective tissue repair.

74. A method, as in claim 73 wherein said adipose tissue is fat tissue.

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75. A method for promoting mammalian connective tissue regeneration using a subset of nucleated cells found in bone marrow, comprising the steps of:

a) isolating said cells by any suitable means,  
20 wherein said means does not involve in vitro culturing;  
and

b) contacting by any suitable means said cells with a site in a patient requiring connective tissue repair.

5 76. A method for promoting bone or cartilage repair at a connective tissue site in a patient requiring repair, comprising the step of surgically implanting a prosthetic device at said site, said device having been seeded with precursor cells having osteogenic and  
10 chondrogenic potential, and wherein said cells have been isolated from peripheral blood.

77. A method for promoting bone or cartilage repair, as in claim 76 wherein said cells have been isolated as  
15 in claim 1, 7, 10, or 11.

78. A method for promoting bone or cartilage repair at a connective tissue site in a patient requiring repair, comprising the step of surgically implanting a  
20 prosthetic device at said site, said device having been seeded with precursor cells having osteogenic and chondrogenic potential, and wherein said cells have been isolated from adipose tissue.



79. A method for promoting bone or cartilage repair, as in claim 78 wherein said cells have been isolated as in claim 17, 18, 19, 20, or 22.

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80. A method for promoting bone or cartilage repair at a connective tissue site in a patient requiring repair, comprising the step of surgically implanting a prosthetic device at said site, said device having been  
10 seeded with precursor cells having osteogenic and chondrogenic potential, and wherein said cells having been isolated from bone marrow without an in vitro culturing step.

15 81. A method for promoting bone or cartilage repair, as in claim 80 wherein said cells have been isolated as in claim 12, 14, 15, or 16.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/01213

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :C12N 5/00, 15/00; A61K 48/00

US CL :514/44; 435, 172.3, 240.1, 240.2, 320.1; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435, 172.3, 240.1, 240.2, 320.1; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS MEDLINE BIOSIS EMBASE CAPLUS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,281,422 A (BADYLAK ET AL) 25 January 1994, see entire document.	1-81
Y	MORITZ et al. Human Cord Blood Cells as Targets for Gene Transfer: Potential Use in Genetic Therapies of Severe Combined Immunodeficiency Disease. Journal of Experimental Medicine. August 1993, Volume 178, pages 529-536, see entire document.	1-81
X -- Y	WO 93/08268 AI (CELLPRO, INCORPORATED) 29 April 1993, see entire document.	1-3, 7-9 ---- 1-81
Y	CA 1,326,836 C (CELLPRO, INCORPORATED) 08 February 1994, see entire document.	1-81

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

10 MARCH 1997

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